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Modeling of the α-lactalbumin and β-lactoglobulin protein separation

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ABSTRACT

This work used the General Rate Model (GRM) to evaluate the experimental data of α-lactalbumin (α-la) and β-lactoglobulin (β-lg) mass transfer using size exclusion chromatography (SEC). The chromatographic simulation has become necessary in large scale production processes. Mathematical models have been used for the optimization and control of different operating conditions of the process, as well as providing calculations for the process scale-up. For the SEC experiments, the aqueous biphasic system was composed of polyethylene glycol 1500 g/mol, potassium phosphate and whey protein isolate. The polymeric phase was enriched with α-la and the saline phase with β-lg. The experiments were conducted using a glass column packed with the Sephadex G-25® gel. Both proteins were quantified by reverse phase liquid chromatography. The experimental data were fitted by non-linear regression, using the successive quadratic programming algorithm. The mass transfer model utilized represented adequately the SEC experimental results.

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1. Introduction

The protein content of cheese whey ranges from 6 g/L to 9 g/L, representing a large available potential (Wit, 1998; Mcintoshi et al., 1998). Cheese whey has the advantage of being a low cost source of protein which does not require pretreatment for large scale processing. One way of reusing the whey proteins for human consumption is to use them in nourishing formulas (Wit, 1998). The potential high added-value and wide applicability, in terms of both quantity and quality, of cheese whey proteins justify the development of separation and purification processes of these biomolecules (Chatterton et al., 2006; Konrad and Kleinschmidt, 2008).

Chromatographic methods are known for their high separation efficiency; this includes the separation of biological products. Large scale preparative liquid chromatography is an important technique for the isolation and purification of biomolecular mixtures such as proteins, peptides, amino acids, enzymes and others (Asenjo, 1990; Fallow et al., 1993; Persson et al., 2004). SEC chromatography is one of the most recognized chromatographic processes used for the purification of proteins. In practice, SEC is used as the final step of the purification process aiming at: (a) changing the mobile phase to another which can be vaporized during a subsequent step (such as lyophilization or concentration) and (b) reducing the amounts of contaminants such as salts, polypropylene, non-ionic detergents and others (Fallow et al., 1993; Sun et al., 2004).

When developing large scale chromatographic processes, it is important not only to obtain a good separation system, but also to reduce operational costs and to increase process reliability. For this reason, modeling emerges as an important step

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Nomenclature

List of symbols

Bi	Biot number of mass-transfer of a solute
C_b	concentration of a solute in the bulk-fluid phase (mol l^{-1})
C_0	initial concentration of the solute (mol l^{-1})
C_p	concentration of a solute in the stagnant-fluid phase inside particle macropores (mol l^{-1})
C_f	feed concentration profile of solute (mol l^{-1})
D_p	effective diffusivity in particle macropores (m s^{-1})
D_b	axial dispersion coefficient (m^{-1})
K_m	film mass transfer coefficient of a solute (m s^{-1})
L	column length (m)
Pe	Peclet number of axial dispersion for a solute
R	radial coordinate for a particle in cylindrical coordinate system
R_p	particle radius (m)
t	dimensional time ($t=0$ is the moment a sample enters a column)
z	Z/L
Z	column axial coordinate in cylindrical coordinate

Greek symbols

η	dimensionless group
ξ	dimensionless constant
ε_a^p	accessible particle porosity
ε_p	particle porosity
ε_b	porosity of the bed
τ	dimensionless time
τ_{inj}	dimensionless time duration for a rectangular pulse of the sample
υ	interstitial velocity (m s^{-1})

in the separation process development since it enables a less problematic scaling up procedure, while also contributing to facilitate optimization and control of the process. The use of computer simulations greatly reduces the number of experiments, diminishing the costs and time required to obtain results (Subramanian, 1998; Luyben, 1989; Persson et al., 2004).

When developing mathematical models, it is often necessary to make some assumptions in order to reduce the complexity of the models, focusing on physical effects and the most important phenomena (Subramanian, 1998). The considerations are not applicable in all cases and for this reason each situation should be treated with the necessary care. For the mathematical SEC model, it can be assumed that:

(a) The column is isothermal.
(b) There is no interaction between the solutes.
(c) Mass transfer and diffusion coefficients are constant.
(d) Column packing is made of spherical particles of a uniform size.
(e) The porosity of the column bed is constant throughout the length of the column.
(f) Radial diffusion in the column can be disregarded.

The GRM is based on the conservation of mass principles and for SEC it considers the existence of axial dispersion in the liquid phase, mass transfer in the film between the phases and solute diffusion into the pores of the particles which make up the column bed (Zhiguo et al., 1998; Kabátek et al., 1997; Goto and Coy, 2000; Altennhöner et al., 1997; Persson et al., 2004; Laatikainen et al., 2007). Eqs. (1) and (2) are based on the GRM:

$$-D_b\frac{\partial^2 C_b}{\partial Z^2}+\upsilon\frac{\partial C_b}{\partial Z}+\frac{\partial C_b}{\partial t}+\frac{3K_m(1-\varepsilon_b)(C_b-C_{p,R=R_P})}{\varepsilon_b R_p}=0 \quad (1)$$

$$\frac{\partial C_p}{\partial t}=D_P\left(\frac{\partial^2 C_P}{\partial R^2}+\frac{2\partial C_P}{R\partial R}\right) \quad (2)$$

Initial conditions for Eqs. (1) and (2) are:

$$t=0 \quad \Rightarrow C_b=C_b(0,R,Z),\quad C_P=C_P(0,R,Z) \quad (3)$$

$$Z=0 \quad \Rightarrow \frac{\partial C_b}{\partial Z}=\frac{\upsilon}{D_b}\left[C_b-C_f(t)\right] \quad (4)$$

$$Z=L \quad \Rightarrow \frac{\partial C_b}{\partial Z}=0 \quad (5)$$

$$R=0 \quad \Rightarrow \frac{\partial C_P}{\partial R}=0 \quad (6)$$

$$R=R_P \Rightarrow \frac{\partial C_P}{\partial R}=\frac{K_m}{\varepsilon_p^a D_b}(C_b-C_{p,R=R_p}) \quad (7)$$

The dimensionless terms are defined as follows:

$$z=\frac{Z}{L};\ \tau=\frac{\upsilon t}{L};\ r=\frac{R}{R_P};\ c_b=\frac{C_b}{C_0};\ c_P=\frac{C_P}{C_0}$$

$$Pe=\frac{\upsilon L}{D_b}\quad Bi=\frac{K_m R_p}{\varepsilon_p^a D_p}\quad \eta=\frac{\varepsilon_p^a D_p L}{\left(R_p^2\upsilon\right)}\quad \xi=\frac{3Bi\eta(1-\varepsilon_b)}{\varepsilon_b}$$

In which C_0 is the initial concentration of the solute, R_p is the particle diameter, R is the radial coordinate of the particle, L is the column length, K_m is the mass transfer coefficient, Pe is the Peclet number, η is a dimensionless number, Bi is the Biot number and ξ is a dimensionless constant. The respective dimensionless forms of Eqs. (1) and (2) for the liquid phase and the particle are:

$$-\frac{1}{Pe}\frac{\partial^2 c_b}{\partial z^2}+\frac{\partial c_b}{\partial \tau}+\frac{\partial c_b}{\partial z}+\xi(c_b-c_{P,r=1})=0 \quad (8)$$

$$\frac{\partial c_p}{\partial \tau}=\frac{\eta}{\varepsilon_p^a}\left(\frac{\partial^2 c_p}{\partial r^2}+\frac{2}{r}\frac{\partial c_p}{\partial r}\right) \quad (9)$$

where the initial and boundary conditions are:

$$\tau=0\Rightarrow \quad c_b=c_b(0,r,z)\ \ c_p=c_p(0,r,z) \quad (10)$$

$$z=0\Rightarrow\frac{\partial c_b}{\partial z}=Pe[c_b-C_f(\tau)],\ \text{where}: C_f(\tau)=\begin{cases}1\Rightarrow 0\le\tau\le\tau_{inj}\\ 0\Rightarrow\tau>\tau_{inj}\end{cases} \quad (11)$$

in which τ_{inj} is the dimensionless time for injection of the sample.

$$z=1\Rightarrow\frac{\partial c_b}{\partial z}=0 \quad (12)$$

$$r=0\Rightarrow\frac{\partial c_p}{\partial r}=0 \quad (13)$$

$$r=1\Rightarrow\frac{\partial c_p}{\partial r}=Bi(c_b-c_{p,r=1}) \quad (14)$$

2. Materials and methods

2.1. Chemicals

All chemicals were of analytical grade and were readily available commercial products. Ultrapure water for all the experiments was obtained from a Milli-Q system (Millipore Inc., MA, USA).

2.2. Aqueous two-phase systems preparation

Aqueous two-phase systems (ATPS) preparation was prepared by mixing appropriate amounts of polyethylene glycol (PEG) stock solutions with a molar mass of 1500 g/mol (50%, Merck, Germany), potassium phosphate (PPP) (30%, pH 7, Merck, Germany) and water (all percentages are by mass). In order to obtain a stock solution of 30% PPP at pH 7, monobasic potassium phosphate (KH_2PO_4) (Merck, Germany) and dibasic potassium phosphate (K_2HPO_4) (Merck, Germany) were weighed (Denver analytical balance, M-310, USA) in accordance with the methodology described by Rojas et al. (2004).

2.3. Purification of proteins present in the different phases

Purification of proteins from the salt and polymeric phases was performed on a semi-preparative scale (ÄKTA *Purifier* 10/100, Pharmacia Biotech). The eluant was monitored by UV absorption at 280 nm. SEC was performed employing a HR10/10® (Amersham Pharmacia) column packed with Sephadex G-25® (Amersham Pharmacia Biotech, Sweden). The maximum pressure of the column bed was 1 MPa. Samples were collected at regular volume intervals with a fraction collector (Frac-900, Pharmacia Biotech). The operating conditions of this process were determined according to those proposed by Rojas et al. (2004).

2.4. Analysis of the fractions obtained from phase purification

The proteins α-la and β-lg retained in the collected fractions were quantified simultaneously using a Shimadzu HPLC system (LC-10VP, Japan) with a LC-10ADVP pump, a SIL-10ADVP autosampler (Shimadzu, Japan) and a SPD-M10AVP photodiode array detector (Shimadzu, Japan) set at 210 nm. Data were analyzed using Class VP5.02 computer software (Shimadzu, Japan). Separation was achieved with a Shim-pack CLC-ODS (M)® C_{18} column (250 mm × 4.6 mm, 5 m particle diameter and 100 Å pore diameter, Shimadzu, Tokyo, Japan) preceded by a guard column of the same material (10 mm × 3.2 mm), temperature of 40 °C, with the operational conditions proposed by Bonomo et al. (2003).

2.5. Mathematical modeling of the SEC

2.5.1. Determination of the porosity of the bed (ε_b)

The porosity of the bed (ε_b) is only dependent on particle size and column packing technique. Evaluation of the exclusion time (t_d) for one molecule of dextran blue (200,000 g/mol) from the bed was used to calculate ε_b.

The value of ε_b was maintained constant for all working conditions of the column, calculated from equation (Altennhöner et al., 1997; Gerberding and Byers, 1998).

$$\varepsilon_b = \frac{t_d 4Q}{\pi d^2 L} \tag{15}$$

in which the mobile phase flow rate (Q) was 4 mL/min, the column length (L) was 10 cm and the column diameter (d) was 1 cm.

2.5.2. Determination of particle porosity (ε_p)

To obtain the particle porosity (ε_p) it is necessary to evaluate the retention time in which one molecule is capable of penetrating the pores of the particles making up the bed. It is also necessary that the molecular mass of this substance is within the working range of the gel. The retention time (t_0) of one molecule of acetone in the column was used in the calculation of ε_p. The equation used was (Zhiguo et al., 1998):

$$\varepsilon_p = \frac{(1 - t_d/t_0)}{(1 - \varepsilon_b)}\varepsilon_b \tag{16}$$

2.5.3. Determination of the accessible porosity of the particle for the solute (ε_a^p)

The accessible porosity of the particle represents a fraction of the accessible volume of the macropore for a particular solute. This parameter was determined for each of the system components: polymeric phase proteins, saline phase proteins, PEG and PPP, in accordance with Eq. (17) (Zhiguo et al., 1998). The retention time (t_R) of the molecules was obtained based on the flow rates used for their separation (4 mL/min and 2 mL/min, for the polymeric and saline phases, respectively).

$$\varepsilon_a^p = \frac{(1 - t_d/t_R)}{(1 - \varepsilon_b)}\varepsilon_b \tag{17}$$

2.5.4. Numerical solution of the model

The mathematical model of the SEC data from Eqs. (8) and (9) together with the respective initial and boundary conditions from Eqs. (10)–(14) were reduced to a single system of algebraic equations, solved using an implicit method of finite differences centralized in space and one step in front of time. This system of differential and algebraic equations was solved using the Gauss–Seidel method (Conceição et al., 1987). Optimal values of *Pe*, *Bi* and η were obtained by applying non-linear regression using a successive quadratic programming method. These two procedures were implemented in a FORTRAN computer program.

3. Results and discussion

3.1. Mathematical modeling of the SEC

The elution profile of the solutes present in the phases was determined from the physical parameters and from the mass transfer parameters (Table 1).

Using the mathematical model developed, the dimensionless concentration of the components was calculated as a function of the dimensionless time. The results are presented in Fig. 1, where the elution profiles of the polymeric and saline phases can be observed, respectively, and compared with the data obtained from the chromatographic determinations. The developed mathematical model was capable of accurately predicting the elution profiles and retention times of the solutes. In all the numerical solutions, deviation of the mass balance was less than 0.1%.

Table 1 – Physical parameters used in the model and predicted mass transfer parameters.

Solute	*Pe*	*Bi*	η	ε_p	ε_b	ε_a^p
Protein saline phase	1850	100	0.0500	0.7477	0.4023	0.0894
Protein polymeric phase	1350	2000	0.0053	0.7477	0.4023	0.1704
Salt (PPP)	4000	7.669	7.5325	0.7477	0.4023	0.8308
PEG	38.60	104.50	332.60	0.7477	0.4023	0.7072

3.1.1. *Determination of the model's physical parameters*

Values of the parameters ε_b, ε_p and ε_a^p, used in the simulation with the developed model, are presented in Table 1. Eqs. (15) and (16) were used to calculate the values of ε_b and ε_p, respectively. From Table 1, ε_b remains constant for all types of solutes since this parameter is only dependent on the particle size of the gel in the column and on the column packing procedure. Because it is a direct function of ε_b, ε_p also remains constant (Zhiguo et al., 1998; Altennhöner et al., 1997).

For each of the macromolecules, values of ε_a^p were calculated by Eq. (17). Proteins showed low ε_a^p values and, due to their elevated molecular mass, they were totally excluded from the particle pore volume (depending on the molecular exclusion limit of the gel). However, due to their low molecular masses, salt and PEG penetrate the particle pores. For this reason their ε_a^p values are greater.

3.1.2. *Calculation of the mass transfer parameters*

The mass transfer parameters (*Pe*, *Bi* and η) were calculated for the flow rate and injection volume of the samples as selected from of the results gotten for Rojas et al. (2004). Thus the used operational conditions had been, 2 mL/min and 0.5 mL for the saline phase and 4 mL/min and 0.5 mL for the polymeric phase.

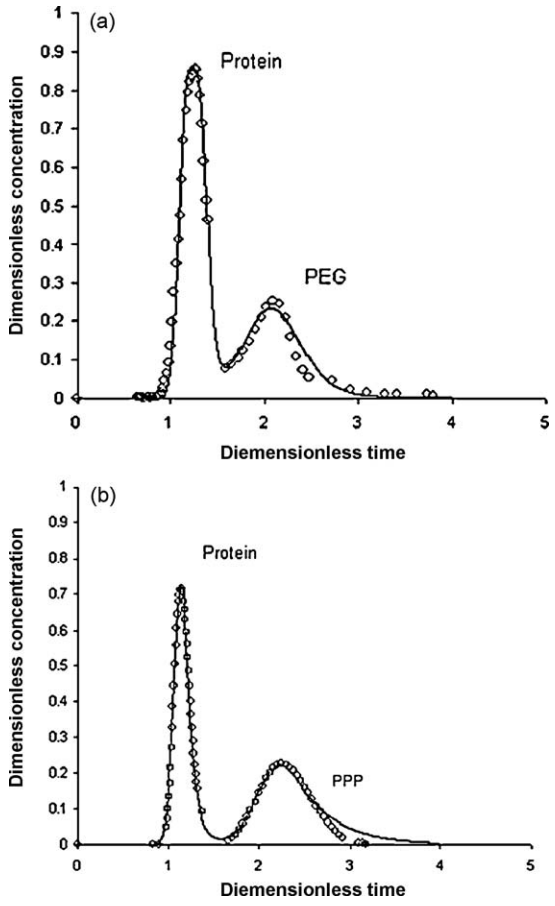

Fig. 1 – Elution profile of the components in the polymeric phase (a) and saline phase (b). Predicted (—) and observed (○).

The mass transfer parameters were obtained for the General Rate Model and presented in Table 1. The value of *Pe* is directly related with the axial dispersion coefficient (D_b) where D_b is a function of both the molecular diffusion and the retrospective mixture. In this work, the diverse solutes developed possessed different molecular diffusion values and consequently, different *Pe* values. The differences in *Pe* are also due to variations in the operational conditions of the phases, principally the flow rate, which exerts the greatest influence (Guiochon et al., 1994). η is related to the diffusion coefficient inside the macropores (D_p). Since D_p depends on the solute's molecular diffusivity, proteins presented low η values which are eluted before salt and PEG. *Bi* is directly related to the mass transfer coefficient of the film (K_m) and inversely with the diffusion coefficient inside the macropores (D_p). The mass transfer coefficient of the film is affected by the hydrodynamic conditions and the flow rate (Guiochon et al., 1994).

3.2. *Influence of the mass transfer parameters on the peak spacing in the SEC model*

Using the developed mathematical model, the effect of the dimensionless parameters *Pe*, *Bi* and η on the mass transfer of the system was evaluated. This sensitivity analysis is important because it allows for the selection of the most stringent parameter when employing the mathematical model.

3.2.1. *Influence of the Pe number*

In order to evaluate the effect of *Pe* in the mass transfer of the four solutes under study, all other parameters were maintained constant. The dimensionless concentration profiles were determined for *Pe* equal to 10, 100, 1000 and 2000. The values used for the other parameters are shown in Table 2.

Simulation results are presented in Figs. 2 and 3. The polymeric rich phase exhibited a similar behavior to the saline phase. Under the evaluated conditions, it could be observed that the increase in *Pe* amplified the resolution of the peaks for the solutes being studied. When *Pe* values were greater or equal to 1000, a minimal influence on the spacing of the chromatographic peaks was observed, thus confirming that *Pe* is inversely related to the axial dispersion coefficient (D_b). Additionally, *Pe* was directly influenced by the mobile phase flow rate. The obtained results agreed with those published by Zhiguo et al. (1998), whom described the insignificant peak spacing for *Pe* values greater than 1000.

3.2.2. *Influence of the Bi number*

To evaluate the influence of *Bi* in the mass transfer of the four solutes under study, all other parameters were maintained constant as shown in Table 3. The dimensionless concentration profiles were determined for values of *Bi* equal to 2, 10, 100 and 200.

The results of the simulations are presented in Figs. 4 and 5. The polymeric rich phase presented a similar behavior to the saline phase. It was concluded that the increase in *Bi* improved

Table 2 – Values of the parameters used in the numerical simulation (effect of *Pe* in the mass transfer).

Solute	*Bi*	η	ε_b	ε_p	ε_p^a	τ_{inj}
Salt (PPP)	10	10	0.4023	0.7477	0.8308	0.16
Protein (saline phase)	10	10	0.4023	0.7477	0.0894	0.16
PEG	10	10	0.4023	0.7477	0.7072	0.16
Protein (polymeric phase)	10	10	0.4023	0.7477	0.1704	0.16

Table 3 – Values of the parameters used in the numerical simulation (effect of *Bi* in the mass transfer).

Solute	*Pe*	η	ε_b	ε_p	ε_p^a	τ_{inj}
Salt (PPP)	500	10	0.4023	0.7477	0.8308	0.16
Protein (saline phase)	500	10	0.4023	0.7477	0.0894	0.16
PEG	500	10	0.4023	0.7477	0.7072	0.16
Protein (polymeric phase)	500	10	0.4023	0.7477	0.1704	0.16

Table 4 – Values of the parameters used in the numerical simulation (effect of η in the mass transfer).

Solute	*Pe*	*Bi*	ε_b	ε_p	ε_p^a	τ_{inj}
Salt (PPP)	500	10	0.4023	0.7477	0.8308	0.16
Protein (saline phase)	500	10	0.4023	0.7477	0.0894	0.16
PEG	500	10	0.4023	0.7477	0.7072	0.16
Protein (polymeric phase)	500	10	0.4023	0.7477	0.1704	0.16

the resolution of the peaks for the studied solutes. The elution profile for *Bi* equal to 200 was equivalent to that of 100. From Fig. 2 it can be observed that the influence of *Bi* on the formation of protein chromatographic peaks within the studied conditions was insignificant. It was also possible to conclude that *Bi* has an inverse relation with the diffusivity coefficient of the particle (D_p). Proteins have a very low D_p value when compared to that of PEG and salt.

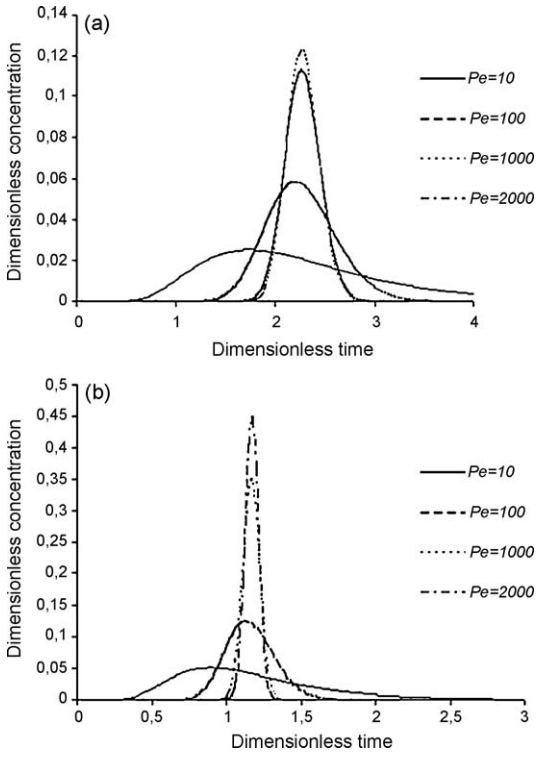

Fig. 2 – Effect of *Pe* on the simulated chromatogram for salt elution in the saline phase (a) and protein elution in the saline phase (b).

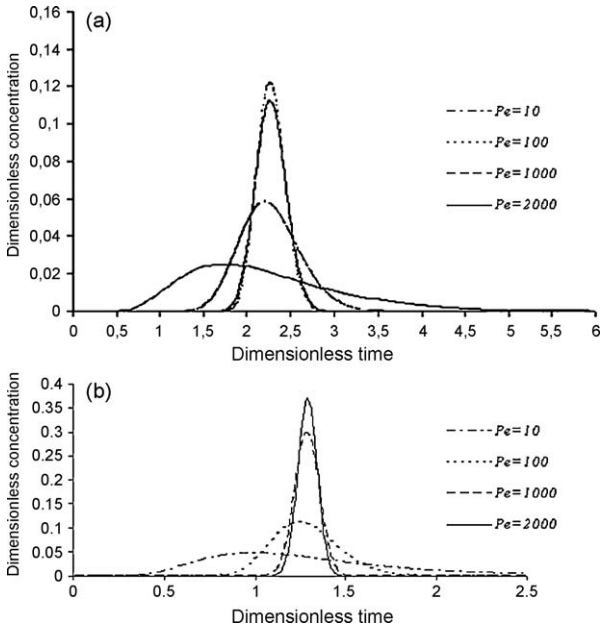

Fig. 3 – Effect of *Pe* on the simulated chromatogram for PEG elution in the polymeric phase (a) and protein elution in the polymeric phase (b).

3.2.3. *Influence of the η parameter*

To evaluate the influence of η in the mass transfer of the four solutes under study, all other parameters were maintained constant as shown in Table 4. The dimensionless concentration profiles were determined for η equal to 2, 10, 100 and 200. The simulation results are presented in Figs. 6 and 7. The polymeric rich phase shown a similar behavior to the saline phase. Those figures show that an increase in η enhanced the peak resolution of the solutes in question. In Fig. 4, it is clear that as η decreases the formation of the peaks is affected, occasionally widening the chromatographic bands. However, within the studied conditions, this influence on peak resolutions of the proteins (Fig. 3b) is very small and when η values are greater or equal to 5, the width of the bands remains practically unchanged. These results are in line with those of Zhiguo

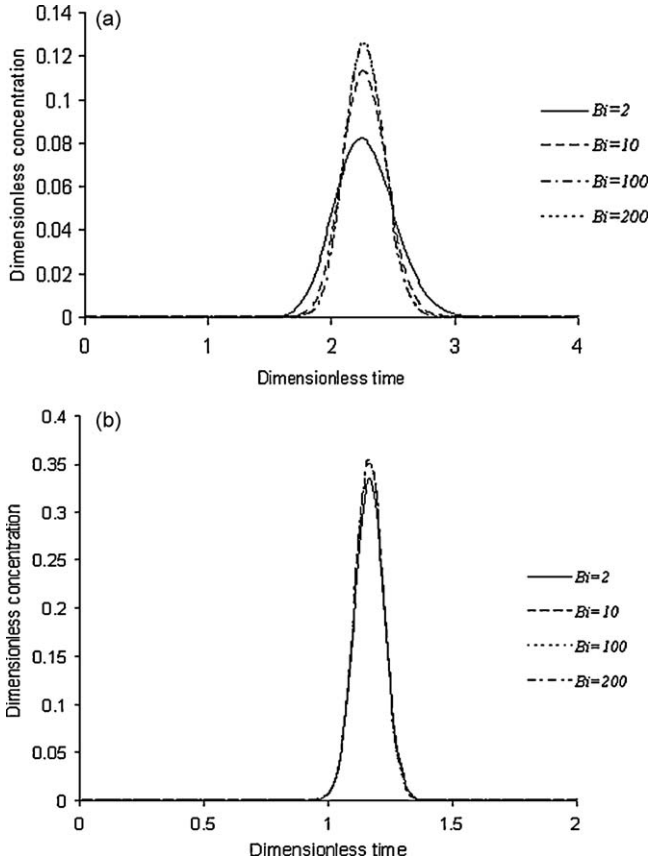

Fig. 4 – Effect of *Bi* on the simulated chromatogram for salt elution in the saline phase (a) and protein elution in the saline phase (b).

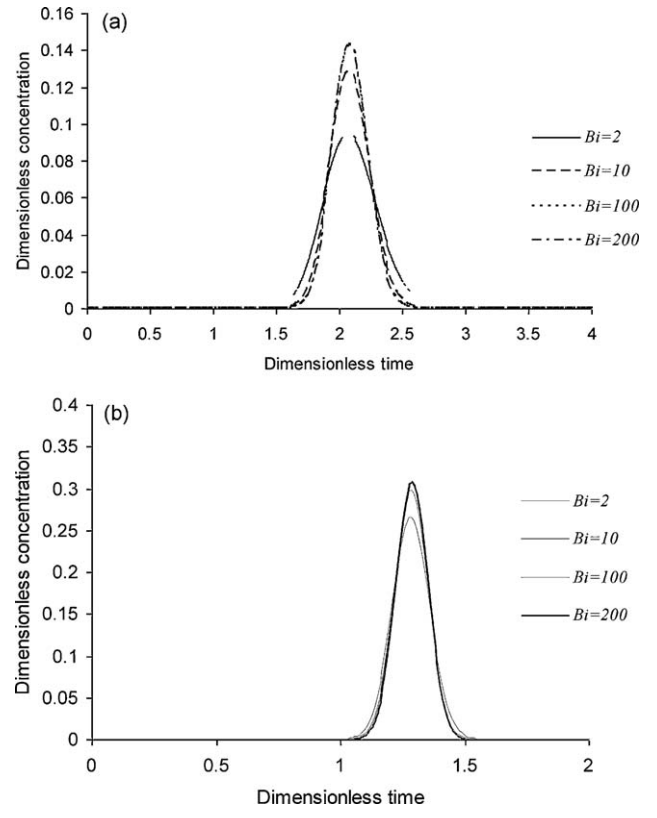

Fig. 5 – Effect of *Bi* on the simulated chromatogram for PEG elution in the polymeric phase (a) and protein elution in the polymeric phase (b).

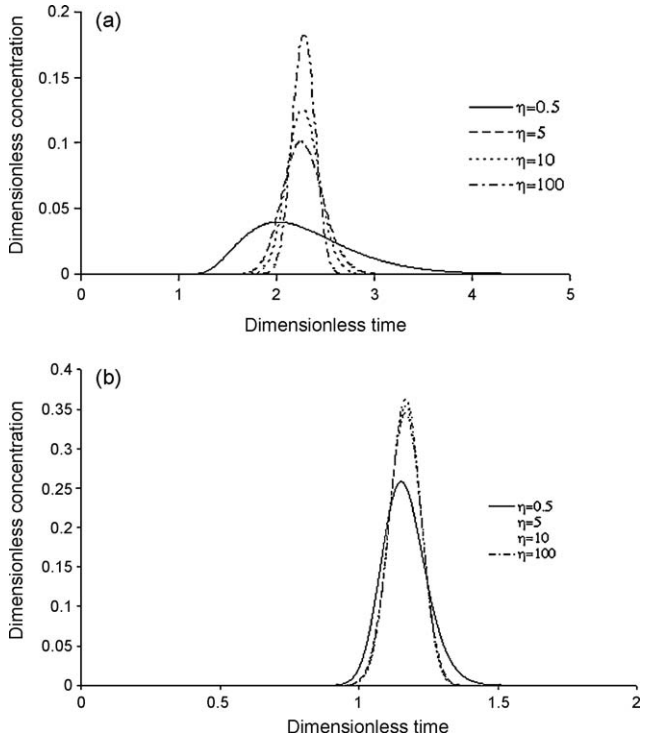

Fig. 6 – Effect of η on the simulated chromatogram for salt elution in the saline phase (a) and protein elution in the saline phase (b).

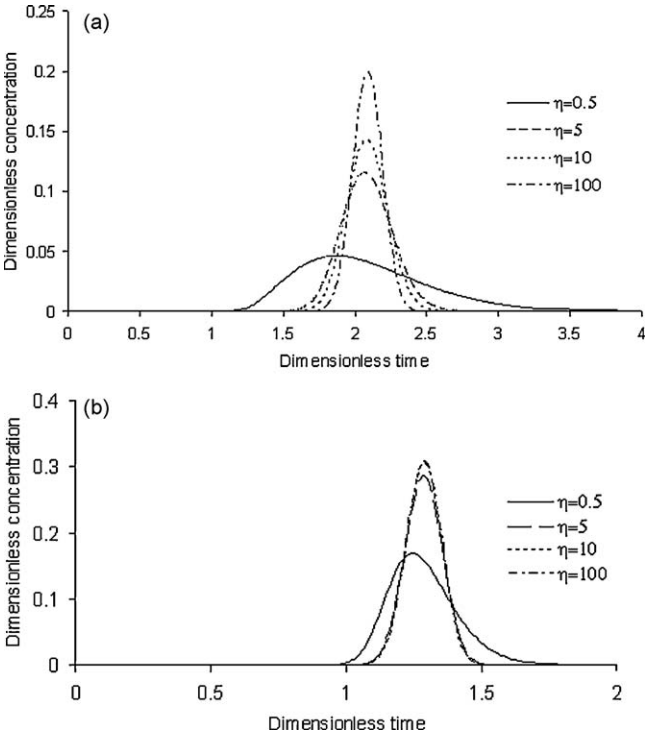

Fig. 7 – Effect of η on the simulated chromatogram for PEG elution in the polymeric phase (a) and protein elution in the polymeric phase.

et al. (1998) who concluded that η significantly effects the formation of chromatographic peaks.

3.3. *Influence of the sample volume injected*

To study the influence of the injected sample volume on the separation of proteins in the utilized column, the following

Table 5 – Values of the parameters used in the numerical simulation (effect of the injected sample volume).

Solute	*Pe*	*Bi*	η	ε_b	ε_p
Salt (PPP)	4000	7.669	7.5325	0.4023	0.7477
Protein (saline phase)	1850	100	0.05	0.4023	0.7477
PEG	38.591	104.467	332.63	0.4023	0.7477
Protein (polymeric phase)	1350	2000	0.005	0.4023	0.7477

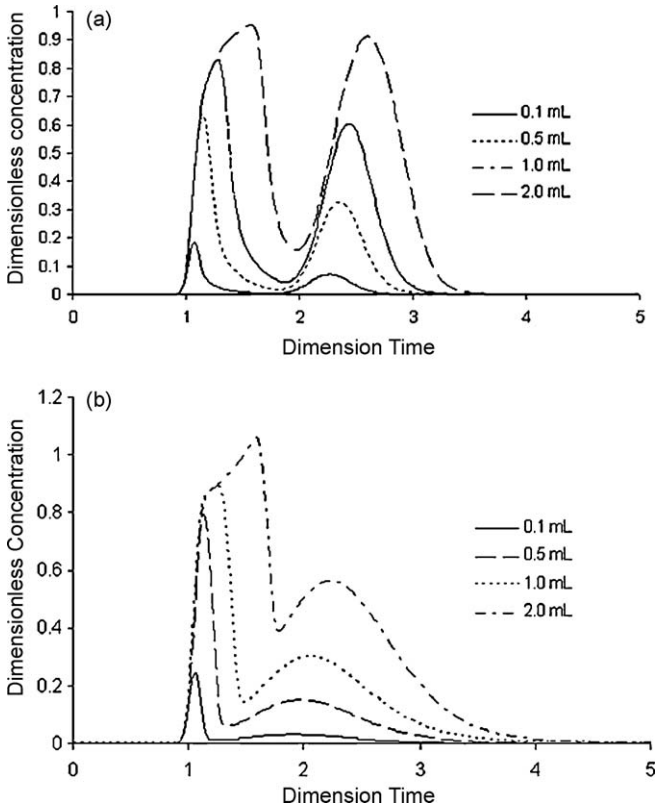

Fig. 8 – Effect of the sample injection volume on the simulated chromatogram for protein elution in the saline phase (a) and polymeric phase (b).

sample injection volumes were considered: 0.1 mL, 0.5 mL, 1 mL and 2 mL which correspond to the fractions of 1.25%, 6.25%, 12.5% and 25% in relation to the total volume of the column. The values of the other parameters used are shown in Table 5. The simulations are shown in Fig. 8. It can be observed that as the sample injection volume increases the separation efficiency decreases; being that the separation resolution of the solutes diminishes as the sample injection volume increases.

Separation techniques for SEC recommend the use of up to 30% of the total column volume (Fischer, 1974), however it can be noted in Fig. 5 that as the injection volume increases, resolution decreases. Felipe and Law (1997) also reported poorer resolution for the separation of cheese whey as sample injection volumes increases.

The results presented in this manuscript show that the prediction of SEC operating conditions by the model is adequate.

4. Conclusions

The mass transfer model used adequately represented the SEC experimental results. Mass transfer coefficients *Pe* and η proved to be the most sensitive in the solution of the mathematical model. The mathematical model provided a satisfactory prediction of both the biomolecule retention times of the process.

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